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The complexity of infectious laryngotracheitis virus: a focus on vaccination and challenge viruses

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The complexity of infectious laryngotracheitis virus: A focus on vaccination and challenge viruses

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DEDICATION

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ABSTRACT

Infectious laryngotracheitis virus (ILTV) causes respiratory distress, decreased egg production, conjunctivitis, and death in chickens. ILT can be prevented by vaccination. Traditionally, that vaccination is with a chicken embryo origin (CEO) vaccine. However, CEO vaccines can revert to virulent ILTV and cause outbreaks. One of the successes of the recombinant vaccines with gene inserts for ILT is that they do not revert to virulence, but do prevent ILT symptoms. However, these same recombinant vaccines do not necessarily prevent replication of ILTV in the trachea of chickens, which then can lead to ILT outbreaks.

One of the regulations for ILT vaccines is that they must protect chickens against inoculation with an ILT challenge virus. All licensed vaccines in the U.S. are licensed by the United States Department of Agriculture's (USDA) Center for Veterinary Biologics (CVB). The CVB provides ILT challenge virus to biologics companies for efficacy testing of vaccines. The current USDA challenge virus strain was generated through egg passage from a 1960s strain of ILTV. Recently, the CVB was able to acquire an ILT field isolate from an outbreak in a laying hen flock that had been vaccinated with a recombinant ILT vaccine.

Mortality, clinical signs and pathogenicity of this field isolate were compared to the current USDA challenge virus. An interesting aspect of the research was that several asymptomatic chickens still had tracheal lesions, both from the field isolate and current challenge virus, indicating viral replication without clinical signs. This is not entirely surprising due to research indicating similar results with some recombinant ILT vaccines,

but it does reinforce that prevention of symptoms does not equal prevention of viral replication.

The ILT field isolate produced clinical signs and tracheal lesions similar to the USDA challenge virus, and the field isolate was able to cause symptoms at a lower infectious douse then the USDA challenge virus to achieve the same results. These results indicate that the ILT field isolate is a viable candidate for use in vaccination challenge studies. Future work will need to be undertaken to perform genetic analysis of the ILT field strain isolated by the USDA and other field strains to determine genetic relationship to vaccine strains.

CHAPTER 1. INFECTIOUS LARYNGOTRACHEITIS AND VACCINATION

Abstract

Infectious laryngotracheitis virus (ILTV) causes respiratory distress, reduction in egg production, conjunctivitis, and death in chickens. Severe cases of infectious laryngotracheitis (ILT) can have morbidity of 100% and mortality rates as high as 70%, with the average percentage for mortality ranging from 10-20%. In mild cases, morbidity can be as low as 5% and mortality can be as low as 0.1%. In the United States, vaccination for ILT is predominately performed using one of the types of vaccines: chick embryo origin (CEO), tissue culture origin (TCO) and recombinant vaccines.

It is critical that traditional live attenuated ILT vaccines prevent disease by preventing infection by wild type strains. However, these vaccines can revert to virulence and cause outbreak situations. The recombinant vaccines prevent disease and do not revert to virulence, but they may allow wild type strains to infect and replicate in birds, which can also lead to outbreaks.

The importance of not only preventing disease, but also infection, has become more apparent as the understanding of the immunological response to ILTV and ILT vaccines has increased. Current research into different vaccination methods, new vectors for recombinant vaccines, and eliciting a cell mediated immune response in the trachea has moved ILT vaccination towards preventing infection.

ILT prevention is a complex challenge. This literature review will give an overview of the ILT virus, advantages and disadvantages associated with currently license vaccines in the U.S, and recent research to improve vaccination for ILT.

1. Introduction

Infectious laryngotracheitis virus (ILTV) causes respiratory distress, reduction in egg production, conjunctivitis, and death in chickens (1). It is an alphaherpesvirus that can cause both severe and mild disease (1-3). Severe cases of infectious laryngotracheitis (ILT) can have morbidity of 100% and mortality rates as high as 70%, with the average percentage for mortality ranging from 10-20% (3). In mild cases, morbidity can be as low as 5% and mortality can be as low as 0.1% (3). Clinical manifestations in mild forms include conjunctivitis, whereas more severe forms may include birds coughing blood from tracheal epithelial cell hemorrhage (3, 4).

In the United States, vaccination for ILT is predominately performed using one of the types of vaccines: chick embryo origin (CEO), tissue culture origin (TCO) and recombinant vaccines (3). This literature review will give an overview of the ILT virus, advantages and disadvantages associated with currently license vaccines in the U.S, and recent research to improve vaccination for ILT.

2. Infectious Laryngotracheitis Virus

2.1. Strain similarities and differences. Discerning differences in ILTV strains has traditionally been performed using restriction fragment length polymorphism (RFLP) analysis. The RFLP analysis divides ILTV strains into different classes based on genetic markers of ILTV, including genes for ICP4, glycoprotein G, glycoprotein M, and thymidine kinase (5-10). Current research has also focused on full genome sequencing to discern strain differences (9, 10).

Data from both RFLP and sequencing indicates that ILTV strains are genetically similar. However, there are distinct differences between American-European strains and Australian strains (8-10). The Serva strain of ILTV is predominately found in vaccines

and has been genetically linked to outbreak strains in Europe and the Americas. The SA2 and A20 strains are predominately found in vaccines and outbreak strains in Australia. This difference in strains by continent has led to variations in RFLP class designation between the American and Australian ILTV strains (6-8, 10).

Although ILTV strains are genetically similar, small genetic differences in herpesviruses can cause dramatic changes in clinical symptoms. The SA2 and A20 strains are 99.9% genetically identical. The SA2 strain is a CEO vaccine in Australia. To reduce the virulence of the SA2 strain, the virus was passed in chicken cells. The TCO vaccine that was produced was designated the A20 strain. Based on full genomic sequencing, SA2 and A20 have two single nucleotide polymorphisms (SNP) that are nonsynonymous, one in ORF B and one in UL15 genes. Amino acid changes in these two genes appear to cause a decrease in cell to cell spread in the A20 strain, and in the process, decrease the virulence of the virus (9).

The importance of the genetic differences between the continental strains was not fully realized until a European vaccine for ILTV was introduced into Australia. Flocks in the same geographical area were vaccinated with the standard Australian strain A20 and the European Serva vaccine strain (8, 11). Following a disease outbreak in this area, two new strains of ILTV were isolated. Based on RFLP analysis, sequencing and phylogenetic analysis, the recovered strains were genetically very similar and were a result of recombination events of the European and Australian vaccine strains. An example of one of the recombination events is that the ICP4 sequence of the Australian strain was replaced with the ICP4 from the American strain (8, 10, 11).

Although this is the first time a significant recombination event was documented causing an outbreak of ITLV, it has happened before (10). Based on phylogenetic analysis outbreaks in production facilities can often be traced back to vaccine strains, however outbreaks in backyard flocks can be caused by virus that is genetically different than vaccine strains. These backyard flock strains have genetic components that are similar to vaccine strains, but also have elements that are different, indicating that recombination of ILTV may happen more often than first thought (10).

2.2. Serological detection. Serologically, all ILTV strains are similar because antiserum produced from one strain can neutralize another. Detection of antibodies to ILTV in chickens is performed using enzyme linked immunosorbent assays (ELISAs) (3). There are currently two licensed diagnostic test kits for ILTV antibody detection from Zoetis (Synbiotics) and Biochek (12).

ELISA assays have also been shown to be as sensitive as other serological tests, including virus neutralization, agar gel immunoprecipitation and indirect immunofluorescence assays (13). It is also an assay that can be automated for high throughput analysis (3). ELISAs using monoclonal antibodies to specific glycoproteins could be valuable in differentiating birds vaccinated with recombinant vaccines from those either vaccinated with CEO or TCO vaccines or infected with a field strain virus (14).

3. ILT vaccination

3.1. CEO vaccines. CEO vaccines are live viruses that have been attenuated by repeated passage in embryonated chicken eggs (3). These vaccines, ideally, do not produce disease, but do cause infection in the trachea, and elicit an immune response

(15). Onset of immunity can occur a few days after vaccination, with duration of immunity lasting fifteen to twenty weeks after vaccination, although it can last up to a year (3).

A problem with CEO vaccines is reversion to virulence (14, 16). Herpesviruses are known to become latent and then reactivate, usually due to an environmental stress (3, 16). CEO vaccines, although attenuated, are live herpesviruses and can become latent. Certain stressors, like moving chickens to new housing or the start of lay, can cause reactivation of latent ILT virus (17). This reactivation can lead to shedding of the virus, and cause horizontal transmission to naïve chickens (14, 18, 19). Once infected, naïve chickens can become symptomatic, leading to an ILT outbreak.

Increased virulence, including mortality, morbidity and tracheal lesions, can occur after one passage of CEO vaccine through a chicken, although morbidity, mortality and tracheal lesions increase considerably after 10 to 20 passages in chickens (16). Due to this reversion to virulence, some states require approval before use of the CEO vaccines for vaccination (20). Approval in Texas is intended to control a confirmed outbreak where all birds to be vaccinated are known to have already been exposed to the virus (20).

3.2. TCO vaccines. TCO vaccines are produced in cell culture to attenuate the virus. On chicken passage studies, increases in morbidity, mortality or lesion scores that were observed for the CEO vaccines, were not noticed in the TCO vaccines (16). According to these same studies, there was a moderate increase in morbidity at chicken passage 20, but no increase in death or lesion scores (16). TCO vaccines do have a decreased duration of immunity when compared to CEO vaccines (3). ILTV growth in

cell culture does not require certain genes that are needed for *in-vivo* replication, including genes in open reading frames (ORFs) A to E (21). This may be why TCO vaccines have a reduced risk of reversion to virulence.

3.3. Administration of CEO and TCO vaccines. CEO and TCO vaccines are administered via drinking water and coarse spray (4, 22-24). They can also be given via infraorbital sinus inoculation (22, 24). Administration of CEO and TCO vaccines occurs mainly in layer or breeder type chickens. Vaccination is not recommended for broiler type chickens unless other birds in the flock are vaccinated or there is an outbreak (22, 24). ILTV vaccination is expensive to implement for broilers and vaccination can cause reduced performance (22). In broiler production systems, biosecurity is seen as the main form of prevention for ILT outbreaks (22, 24).

For layer or breeder production systems, CEO and TCO vaccines are administered at 6 to 8 weeks of age, and then again at 12 to 15 weeks (24). The greatest protection occurs from 15-20 weeks, and protection immunity may vary over the year (3). The effectiveness of revaccination in boosting immunity is unknown (3).

3.4. Recombinant vaccines. Another option for ILT vaccination is recombinant vaccines. Licensed recombinant vaccines do not revert to virulence like the CEO vaccines since they only contain one or two gene inserts from ILTV that are inserted into herpes virus of turkeys (HVT) or fowl pox vector (3, 14, 15, 25, 26). Vaccination can occur early by *in ovo* vaccination or at 1 day of age by subcutaneous vaccination. *In ovo* vaccination provides protection at a very young age, unlike the traditional live attenuated vaccines, where vaccination may not occur until 6 weeks of age (14, 26).

The recombinant vaccines depend on the immunogenicity of herpesvirus glycoproteins. The main glycoproteins used in research are glycoproteins B, C, D G and I (2, 15, 27-32). Currently licensed vaccines contain glycoproteins B, D or I, or a combination of these glycoproteins (25). The importance of expression of glycoproteins for ILT disease prevention is discussed more in depth later in this paper.

4. Recombinant vaccine regulation in the United States

As of January 2015, 31 poultry products licensed in the US included a recombinant agent (12). In the early 1990s, the first poultry recombinant vaccine was licensed. It used fowl pox as a vector for a Newcastle Disease Virus (NDV) gene insert (33). Shortly after, a fowl pox-ILTV recombinant vaccine was licensed. This vaccine is produced by Ceva Biomune. There are also HVT-ILTV recombinant vaccines from two separate manufacturers, Ceva Biomune and Merck (12).

The USDA's Center for Veterinary Biologics (CVB) licenses all animal vaccines in the United States, including recombinant vaccines. The CVB was created to enforce the Virus Serum Toxin Act and regulations for vaccine licensing and testing are detailed in part 9 of the Code of Federal Regulations (9CFR). These regulations include testing requirements for purity, potency, efficacy and safety for both killed and live standard type vaccines (34-36).

Licensure of recombinant vaccines entails complexities that the original creators of the 9CFR could not have anticipated. Some of those include: stability of the gene insert or inserts; safety of a genetically modified live product in the field; production of recombinant antigenic protein; and purity of the genetic sequences of both the vector and the inserts (35).

General guidelines for licensure of biologics products using new biotechnology were issued in USDA Veterinary Services (VS) Memorandum 800.68 in 1984 (37). These basic guidelines were an attempt to reconcile the USDA requirements with National Institutes of Health standards on biotechnology research. After the release of the first recombinant vaccine for pseudorabies, there was concern from the public about the safety of live genetically modified vaccine organisms being released into the environment and mixing with wild type organisms. In response to that concern, the USDA outlined new procedures incorporating the National Environmental Policy Act and requiring environmental assessment of the impact of recombinant products (35).

VS Memo 800.205 outlines the studies and testing required for varying categories of biotechnology derived veterinary products beyond typical 9CFR testing (38). As outlined in VS Memo 800.205, testing may include recombination studies, shed and spread studies and sequencing of the construct, flanking regions of the insert, and the insert(s). If a recombinant construct will be expressing an antigenic protein, this protein should be detectable by an *in vitro* assay (Western Blot, fluorescent antibody staining, etc.). The memo creates three different categories of biological products: Category I are biotechnology derived products that are inactivated; Category II are gene deleted, live products; and Category III are live vector products with foreign gene insert(s). The testing required for a recombinant product depends on the construct, what category of biological product, and a risk analysis performed by the CVB (38).

For ILT recombinant vaccines, the vaccine has to perform to the same efficacy standards as the traditional ILT vaccines. This efficacy standard is tied to a vaccination challenge study described in 9CFR 113.328 (39). The vaccination challenge study

involves vaccinating 20 birds and then challenging them with a virulent ILT virus, which can be provided by the CVB. The birds are monitored for ten days for ILT disease symptoms. Protection is measured based on decrease of symptoms of the disease when compared to non-vaccinated, ILTV challenged control birds (39).

5. ILT recombinant vaccination problems

Protection from ILT disease by vaccination with ILT recombinant vaccines depends on a humoral immune response and antibody production to the ILTV glycoprotein inserts. However, it was determined that ILTV immunity is driven by cell mediated immunity and not humoral immunity. Bursectomized chickens inoculated with traditional CEO ILT vaccine were protected from ILT challenge virus infection (40). This indicates that antibodies to ILT do not play an integral role in preventing ILTV infection.

More recently, studies have indicated that the live CEO vaccines produce an immune response that prevents viral replication upon challenge (14, 15, 25). It appears that the initial T- cell response upon vaccination in the trachea is critical to prevent future replication of virus, specifically a T-helper cell response (15, 31, 41, 42). The HVT-ILTV and fowl pox-ILTV recombinant vaccines are not given by an oral-pharyngeal route. The recombinant vaccines depend on the humoral immune response, not the cell mediated immune response of the trachea. Antibodies can prevent symptoms of a field-acquired ILT infection, but virus replication can continue, which can lead to viral latency. As mentioned previously, upon stress, the latent virus can be reactivated and disease outbreaks can occur.

6. Future of ILT recombinant vaccines

6.1. Dual vaccination. Continued viral replication, leading to decreased efficacy, has also been reported for recombinant vaccines with Newcastle Disease Virus (NDV) inserts. The solution that was found was to vaccinate with the recombinant NDV vaccine and boost with the live attenuated vaccine. This produced better protection than using either vaccine alone (43, 44). This same method may be useful for vaccinating for ILT, especially in relation to the TCO vaccines. The immune system could be primed, using the recombinant vaccine, for vaccination using the lower virulence TCO vaccine. Further research would have to be performed to determine if protection levels would be comparable to the NDV vaccination study.

6.2. Immune modulator and DNA vaccine. One of the ways for a recombinant ILT vaccine to induce a humoral immune response is by expression of an immune modulator protein. Researchers have demonstrated this using a fowl pox vector expressing ILT glycoprotein B (gB) and interleukin-18 (IL-18) (41). Glycoprotein B was chosen because it is known to be conserved among herpesviruses. It also has been used in previous recombinant ILTV vaccines and has been shown to prevent disease. IL-18 was chosen because it stimulates T-helper 1 cells to secrete interferon-γ (IFN-γ) (31, 41, 42). IFN-γ has been shown to be an important signaling cytokine for preventing herpesvirus infection, clearing herpesvirus infection and preventing herpesvirus from reactivating from latency (45-48).

The fowl pox-gB-IL-18 (rFPV-gB/IL-18) vaccine increased CD4 to CD8 T- cell ratios significantly in comparison to vaccination with recombinant fowl pox containing only a glycoprotein B insert (rFPV-gB). After challenge, morbidity and mortality were

0% for the rFPV-gB/IL-18 vaccine, which was significantly less than for that of the rFPV-gB or ILTV live attenuated vaccines. Also, 0 out of 10 birds had detectable ILTV by PCR of tracheal tissue for the rFPV-gB/IL-18 vaccine, whereas 2/10 and 3/10 birds did for the rFPV-gB and ILTV live attenuated vaccines respectively. The rFPV-gB/IL-18 vaccine protected 100% of birds based on clinical signs, mortality and PCR of tracheal tissues (41).

An immune modulator can also be used with DNA vaccines. DNA vaccines are naked DNA that are encoded with an immunogenic protein or proteins, that when inoculated into cells or an animal, produce the immunogen. These vaccines do not involve injecting live virus and therefore there is no worry of reversion to virulence.

A DNA vaccine containing genes for ILT gB and IL-18 caused significantly higher IFN-γ levels in peripheral blood mononuclear cells (PBMCs) of chickens than did a DNA vaccine expressing just the gB plasmid. The gB/IL-18 DNA vaccine had both morbidity and detectable virus in the trachea; although the level was significantly lower than the DNA vaccine expressing just the gB plasmid. The gB/IL-18 vaccine had an 80% protection rate based on clinical signs, mortality and PCR of tracheal tissue, but did require a booster vaccination 14 days after initial vaccination (31, 42).

6.3. Other vectors. Another way to trigger the cell mediated immune response is to use a vector that has an inoculation route similar to ILTV. NDV live attenuated vaccines are typically given by coarse spray or drinking water, similar to how ILTV live attenuated vaccines are administered. Researchers at the Southeast Poultry Research Laboratory (SERPL) created two separate NDV LaSota strain recombinant vaccines, one with an ILTV glycoprotein B (gB) insert and the other with an ILTV glycoprotein D (gD)

insert. The gB and gD vaccines were given at 1 day of age to specific pathogen free egg type chickens and the gB at 3 weeks of age to broiler type chickens (25).

The NDV LaSota strain with either the gB or gD insert were as protective as traditional NDV vaccines against challenge with NDV. When challenged with ILTV, the vaccinated birds from both groups were 90% protected against clinical symptoms, and the birds that did show clinical symptoms had very mild symptoms. By a real time PCR assay, both vaccines decreased viral load in tears and tracheas, with the gB vaccine performing better. The gB vaccine performed as well as the traditional CEO vaccine in broilers, although it did not decrease viral load in the tracheas as much as the live attenuated ILTV vaccines (25).

7. Conclusion

Infectious laryngotracheitis is a major issue for the poultry industry. As an example, a 1998 outbreak in the Delmarva peninsula in the Eastern United States cost producers over $1 million dollars, factoring in decreased production, mortality, vaccination and medication (4). It is also a complex issue, since vaccination can be a major factor in outbreaks.

It is critical that traditional live attenuated ILT vaccines prevent disease by preventing infection by wild type strains. However, these vaccines can revert to virulence and cause outbreak situations. The recombinant vaccines prevent disease and do not revert to virulence.

Importantly, the focus of vaccination is largely on prevention of disease and not prevention of viral infection. The USDA licensed recombinant vaccines for ILT protect against disease symptoms. They meet all of the regulations for an ILT vaccine and meet

the regulations for recombinant vaccines. However, they do not necessarily stop infection by wild type strains. This can lead to wild type virus becoming latent and reactivating at a later time, which can cause outbreak situations.

The importance of not only preventing disease, but also infection, has become more apparent as the understanding of the immunological response to ILTV and ILT vaccines has increased (14, 15, 49). For some viruses, preventing disease may be adequate, but for a herpesvirus like ILTV, that can become latent and reactivate at a later time point, preventing infection is also important.

The future of ILT vaccine research lies in finding a vaccine that does not revert to virulence, and can prevent viral replication upon challenge. The solution may be in performing dual vaccinations with recombinant vaccine and modified live vaccines, expression of an immune modulator in a recombinant vaccine, or looking at different vectors for ILT gene inserts. Due to the complexity of the problems and issues discussed here surrounding ILT vaccination; the solution will need to be a collaborative effort between poultry producers, researchers, vaccine regulators and vaccine manufacturers.

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CHAPTER 2. COMPARISON OF THE PATHOGENICITY OF THE USDA CHALLENGE VIRUS STRAIN TO A FIELD STRAIN OF INFECTIOUS LARYNGOTRACHEITIS VIRUS.

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Abstract

Infectious laryngotracheitis virus (ILTV) causes respiratory disease in chickens. This alphaherpesvirus infects laryngeal tracheal epithelial cells and causes outbreaks culminating in decreases in egg production, respiratory distress in chickens and mortality. There are several different vaccines to combat symptoms of the virus, including chicken embryo origin, tissue culture origin and recombinant vaccines. All vaccines licensed for use in the U.S. are tested for efficacy and potency according to U.S. federal regulation using a vaccine challenge assay involving the use of an ILT challenge virus. This challenge virus is provided to biologics companies by the Center for Veterinary Biologics (CVB), United States Department of Agriculture (USDA). The current USDA challenge virus originated from a vaccine strain and has been subjected to multiple passages in eggs, and may not represent what is currently circulating in the field. The objective of this study was to evaluate and compare the pathogenicity of USDA's challenge virus strain to the pathogenicity of a recent ILT field isolate.

Using the challenge virus and various dilutions of the field isolate, clinical signs, mortality and pathology were evaluated in chickens. Results indicate that the field isolate at a 1:20 dilution is comparable in pathogenicity to the USDA challenge virus at a 1:4 dilution, and that the ILTV field isolate is a viable candidate that could be used as a

challenge virus when evaluating vaccine efficacy.

1. Introduction

Infectious Laryngotracheitis (ILT) is a respiratory disease of chickens which is caused by an alphaherpesvirus and classified as Gallid herpesvirus 1. Disease manifestations include respiratory distress, conjunctivitis, and a decrease in egg production and mortality (1, 2). Infectious laryngotracheitis virus (ILTV) infects laryngeal and tracheal epithelial cells causing lesions in the trachea of chickens, which can lead to mucous build up, tracheal hemorrhaging, suffocation and death. Direct transmission occurs when naïve birds come in contact with infected birds that are in respiratory distress or exhibiting conjunctivitis. As with many herpesviruses, ILTV can become latent in birds that survive infection and be reactivated from latency and transmitted to asymptomatic birds (2). Indirect transmission is caused through inanimate objects, including personnel, equipment, and contaminated litter, coming into contact with infected flocks and then passing the virus on to naïve flocks.

Measures to control ILTV transmission in poultry houses include biosecurity and vaccination programs (1). Vaccination programs often rely on the use of live virus chicken embryo origin (CEO) vaccines, which can revert back to virulence and cause disease outbreaks (2, 3). Risk of reversion to virulence of CEO vaccines has led to increased interest in recombinant vaccines (1). Predominantly, ILTV recombinant vaccines are fowl pox or herpesvirus of turkeys (HVT) vectored with single or multiple glycoprotein inserts (4-6).

All ILT vaccines currently licensed in the U.S. are live viral vaccines, whether they are CEO, tissue culture origin (TCO) or recombinant. CEO and TCO vaccines are attenuated through passage in eggs or in tissue culture.

Although recombinant ILT vaccines are widely used, they may have limitations in providing protective immunity when compared to traditional vaccines (7, 8). Effective protection from ILTV replication in the trachea requires a humoral and cell mediated response (9). Traditional CEO and TCO ILT vaccines, are administered via eye drop or oral routes (i.e. inoculating drinking water) causing the antigen to come into contact with the trachea epithelium. Vaccination triggers a cell mediated response from the immune system and prevents replication and latency of field strains of ILTV (7-9). HVT recombinant vaccines are administered via *in ovo* or subcutaneously at hatch, or fowl pox vectored vaccines are delivered via wing web puncture. Recombinant ILT vaccines may not induce a strong cell mediated immune response in the respiratory tract of chickens (4, 8, 9).

All ILTV vaccines are evaluated for efficacy and potency using vaccination-challenge studies in accordance with Title 9, Code of Federal Regulations (9 CFR), Part 113.328, using a challenge virus provided by the United States Department of Agriculture's (USDA) Center for Veterinary Biologics (CVB) (10). The present USDA ILT challenge virus originated from a 1960's vaccine isolate and may not be representative of currently circulating field strains. If so, vaccine strains that protect against the USDA's challenge virus may not confer sufficient protection against all circulating field strains. The objective of this study was to evaluate and compare the

pathogenicity of USDA's challenge virus strain to the pathogenicity of a recent ILT field isolate.

2. Materials and Methods

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the CVB and National Veterinary Services Laboratories (NVSL) Animal Care and Use Committee.

2.1. Challenge viruses. The present USDA ILT challenge virus (Lot 07-3) originated from a vent brush ILT vaccine from the 1960's. A challenge virus was produced by diluting the previous USDA ILT challenge virus (Lot 86-3) 1:100 in tryptose phosphate broth (TPB) (Media # 10426, NVSL, Ames, Iowa) with penicillin-streptomycin (minimum 23,040 IU/mL penicillin G potassium salt and 72,000 IU/mL streptomycin, Media # 30044, NVSL, Ames, Iowa) used at 2% (2 mL per 100 mL). From this dilution, 0.2 mL was inoculated into the allantoic cavity of 11 day old specific pathogen free (SPF) embryonated chicken eggs (Charles River Laboratories, Wilmington, Massachussetts). At four days post-inoculation, chorioallantoic membranes (CAMs) were harvested and frozen at -70^{o}C. Infected CAMs were thawed and added to an equal amount of TPB and ground using a Lourdes grinder and Tenbroeck glass manual tissue grinder. After grinding, the homogenate was filtered through 4-5 layers of course gauze. Approximately 250 mL of homogenate was collected and then brought to a one liter volume using TPB with 2% penicillin-streptomycin and 1.5 mL was placed into glass cryules using a Cornwall syringe. Cryules were sealed using a heat sealer and stored at -120^{o}C.

The field isolate (lot 11-11) used in this study was recovered from tracheas of egg-type chickens submitted to Iowa State University's (ISU) Veterinary Diagnostics Laboratory (VDL) (Ames, IA). ILT was diagnosed in these chickens based upon clinical signs and the presence of intranuclear inclusion bodies in tracheal epithelial cells. Laying hens in the flock of origin had been previously vaccinated with a recombinant ILT vaccine. The tracheas were acquired from ISU's VDL by the CVB to potentially make new challenge virus because the tracheas were locally available and the flock had been previously vaccinated for ILT by a vaccine other than a CEO vaccine.

Harvested tracheas were ground in 11 mL of 10T antibiotic media (Media # 10411, NVSL, Ames, Iowa) and centrifuged at 1135 g for 20 minutes at 4^{o}C. Three hundred µl of supernatant were inoculated into 11-day-old SPF embryonated chicken eggs (Charles River Laboratories, Wilmington, Massachussetts). ILT virus was propagated on the CAM for three serial passages. After the final passage, CAMs were harvested, ground in TPB (Media # 10426, NVSL, Ames, Iowa) with 2% penicillin-streptomycin (Media # 30044, NVSL, Ames, Iowa), filtered through two layers of course gauze, diluted 1:1 in TPB, bottled in glass cryules (Wheaton, Millville, New Jersey) and stored at -120^{o}C in a vapor phase liquid nitrogen freezer (ThermoScientific, Asheville, North Carolina).

2.2 Testing for extraneous agents. Both the USDA challenge virus and the ILT field isolate were tested for poultry extraneous agents in the standard manner as master seed viruses as laid out in the 9CFR or in USDA Veterinary Services (VS) memorandum. Extraneous agents tested for included anaerobic and aerobic bacteria (9CFR part 113.27), fungus (9CFR part 113.27), mycoplasma (9CFR part 113.28), hemagglutinating viruses

(9CFR part 113.34), avian leukosis virus (9CFR part 113.31), reticuloendotheliosis virus (VS Memo 800.88), and chicken anemia virus (VS Memo 800.89) (11-17).

2.3. Virus Titration. Titrations of USDA challenge virus and the field isolate were performed in 11 day old specific pathogen free embryonated chicken eggs (Charles River Laboratories, Wilmington, Massachusetts). Ten-fold dilutions (10^{-1} to 10^{-6}) of the challenge virus and field isolate were prepared in TPB and 0.2 ml was inoculated via the CAM inoculation route. Six eggs per dilution were inoculated and after seven days the CAMs were scored for typical ILT lesions. The 50% egg infectious dose (EID_{50}) was calculated using the Reed-Muench method (18). Egg titrations were performed on both the USDA challenge virus and field isolate after bottling and freezing, with challenge virus having an EID_{50} of $1 \times 10^{4.1}$ and the field isolate having an EID_{50} of $1 \times 10^{3.4}$.

2.4. Experimental Design. Ninety White Leghorn specific pathogen free chickens (Charles River Laboratories, Wilmington, Massachusetts) were randomized into eight treatment groups at approximately 7 days of age and housed in separate isolator cages (PLAS Labs, East Lansing, Michigan) by treatment group (Table 1). The negative control group and six treatment groups inoculated with the ILT field isolate contained 10 chickens. Twenty chickens were inoculated with USDA's current ILT challenge strain. Twenty chickens were used for the USDA challenge virus to give a larger number of birds to compare to the field isolate. Each bird was given a unique leg band to allow for clinical signs and pathological tracking by bird.

Viruses were diluted in TPB, and at 34 days of age, each bird was inoculated intratracheally with 0.5 mL of appropriate inoculum based on treatment group (Table 1). The scientist performing chicken inoculations was unaware of the virus origin and

dilution. After inoculation, the virus solutions used to make the dilutions were titrated in eggs to determine the EID_{50}, with the USDA challenge having a value of $1 \times 10^{3.5}$ and field isolate having a value of $1 \times 10^{2.8}$. From those values, the EID_{50} was calculated for each treatment group for both the field isolate and USDA challenge virus (Table 3).

2.5. Scoring of Clinical Signs. After inoculation, birds were observed independently, twice daily, by two observers for ten days, and symptoms were scored according to criteria modified from previous work (Table 2) (19). Observers were blinded to the identity of each treatment group. Chickens demonstrating hemorrhaging or severe dyspnea were humanely euthanized, necropsied and tracheas were harvested. On day 10, the remaining birds were humanely euthanized, necropsied and tracheas harvested. A 2-4 cm section of upper trachea, approximately 2-3 cm from the larynx and a 2-4 cm section of lower trachea approximately 1-2 cm from the syrinx were removed and placed in buffered formalin solution. Tracheas were sent to NVSL for pathological scoring by a pathologist who was also blinded to the identity of the treatment groups.

2.6. Effective Dose. Chickens were classified as positive if they had any of the following three characteristics: death, multiple symptoms seen multiple days in a row, or multiple clinical signs seen by both observers. A binomial generalized linear model was fit to the data using a logit link function (logistic regression), $\ln(\pi/(1-\pi)) = \alpha + \beta*(-\log_{10}$ dilution) (20). Maximum likelihood estimates were used in estimating the $\log_{10}$ effective dose 80 (ED_{80}) as $\ln(4) - \alpha/\beta$, where ln represents the natural logarithm. The results were back-transformed and presented as the ED_{80} (20).

2.7. Histological sample processing. Transverse sections of trachea were fixed in neutral buffered formalin, processed and embedded in paraffin, sectioned to 3 µm thickness, mounted on glass slides, and stained with hematoxylin and eosin.

2.8. Histopathological scoring. Tracheal lesions were scored using a scoring system (Table 2) modified from a previous work (21). In the present study, tracheal lesions reflect lesions at the time of death, whether death occurred naturally, at the humane endpoint, or at the end of the study. Scoring criteria reflect not only the progression of acute inflammatory changes, but also takes into consideration more chronic regenerative changes seen in birds that have been known to recover from infection with the virus. Lesion severity was based on loss of mucosal integrity, so that lesions demonstrating sloughing and ulceration were given higher scores than lesions in which the mucosa was covered by a thickened, but intact, regenerating epithelium (Table 2). A bird was considered positive with a tracheal lesion score of 2 or higher. Tracheal lesion scores of 4 or 5 were considered severe.

3. Results

3.1. Pathogenicity. Clinical signs observed included conjunctivitis, mild dyspnea, severe dyspnea, and hemorrhaging from the oropharyngeal cavity and nostrils. Most signs were observed at 4-6 days post-inoculation, except for conjunctivitis, which was observed 5-10 days post-inoculation. Several birds from virus inoculated groups were able to recover from dyspnea, depression and conjunctivitis.

Death or euthanasia due to severe symptoms occurred in most chickens at 4 to 5 days post-inoculation. This is similar to results previously reported by researchers using other strains of ILTV (19, 21). The number of birds showing clinical signs, those

euthanized or chickens that died, and the percentage of positive birds in each treatment group are summarized in Table 3.

All dilutions from both the USDA challenge virus and the field isolate had at least one bird either die or show severe enough symptoms to be euthanized. At the dilution of 1:4 (EID_{50} titer $1 \times 10^{2.6}$), the present USDA challenge virus produced 85% positive birds. This was slightly better than the 80% positive rate caused by the field isolate at dilutions of 1:4, 1:10 and 1:50 (EID_{50} of $1 \times 10^{1.9}$, $1 \times 10^{1.5}$, and $1 \times 10^{0.8}$). Field isolate dilutions of 1:20, 1:100, and 1:250 produced positive rates of 90%, 50%, and 40%, respectively. The ED_{80} of the field isolate was estimated as 14.4 (95% CI: 3.3, 61.6).

The present USDA challenge virus had a mortality rate of 35%. Similar mortality rates were seen for the field isolate at dilutions of 1:4, 1:20, and 1:50, which produced mortality rates of 30%, 40%, and 30%, respectively.

3.2. Tracheal Lesion Scores. Control birds had normal to slightly hyperplastic tracheal mucosa (Fig. 1a). Infected birds demonstrated a variety of lesions ranging from slightly hyperplastic through epithelial syncytia formation and sloughing to complete ulceration with a hemorrhagic and cellular exudate (Fig. 1b-1f). Occasional birds had intact, occasionally cystic, thickened epithelium interpreted as regeneration (Fig. 1d).

Upper and lower tracheal lesion scores for each experiment chicken, the percentage of birds in each group with positive lesions and the percentage of birds in each group with severe lesions is summarized in Table 4. The USDA challenge virus at a 1:4 dilution (EID_{50} of $1 \times 10^{2.6}$) produced upper tracheal lesions in 95% of chickens and in 35% of these birds lesions were severe. In the lower trachea, the USDA challenge

virus caused lesions in 85% of inoculated chickens and in 35% of the birds lesions were severe.

Field isolate dilutions of 1:20, 1:50, 1:100, and 1:250 produced severe lesions in the upper trachea in 40%, 50%, 20% and 20% of the birds, respectively. For both the 1:20 dilution (EID_{50} of $1 \times 10^{1.2}$) and 1:100 dilution (EID_{50} of $1 \times 10^{0.5}$) 100% of the birds had positive tracheal lesion scores in the upper trachea. The dilution resulting in the highest percentage of severe lesions in the upper trachea, at 50%, was the 1:50 dilution (EID_{50} of $1 \times 10^{0.8}$) of the field isolate. Field isolate dilutions of 1:20, 1:50, 1:100, and 1:250 produced severe lesions in the lower trachea in 30%, 50%, 20%, and 20%, respectively. The 1:50 dilution of the field isolate also had 90% positive upper tracheas, as did dilutions 1:4, 1:10, and 1:50 (EID50 of $1 \times 10^{1.9}$, $1 \times 10^{1.5}$, and $1 \times 10^{0.8}$).

In the lower trachea 50% of birds inoculated with the 1:50 dilution had severe lesions; however the number of positives was lower, at 80%, when compared with the upper trachea lesion scores. The dilutions of the field isolate at 1:10 and 1:100 (EID50 of $1 \times 10^{1.5}$ and $1 \times 10^{0.5}$) also had a similar decrease in the number of positives, to 80%, for the lower trachea. The 1:4 and 1:20 dilutions (EID_{50} titers of $1 \times 10^{1.9}$ and $1 \times 10^{1.2}$) of the field isolate had decreases in lesion scores to70% positive for the lower trachea.

4. Discussion

According to 9 CFR 113.328 regulations regarding ILT vaccine testing, at least 80% of the non-vaccinated birds must show clinical signs and/or death for the challenge study to be valid (10). Infectivity studies conducted in 2007 using birds that had been inoculated with the USDA challenge virus lot 07-3 showed 90% of chickens symptomatic for ILT at a 1:10 dilution in 2007. In 2009, a biologics company reported to the CVB

that the USDA challenge virus was not causing clinical signs and/or mortality in 80% of birds. This observation was confirmed by further bird testing by CVB using a 1:10 dilution of challenge virus. The recommendation for using the USDA challenge virus was changed to a dilution of 1:4 (EID_{50} of $1 \times 10^{2.6}$). The apparent reduction in virulence of the USDA's challenge in chickens initiated the search for a new challenge virus.

This study indicates the challenge virus presently used by the USDA will still cause clinical signs and/or death in over 80% of birds at the 1:4 dilution. The field isolate virus performed to regulatory standards at all dilutions except 1:100 (EID_{50} of $1 \times 10^{0.5}$) and 1:250 (EID_{50} of $1 \times 10^{0.1}$). Although the field isolate was prepared slightly differently than the USDA challenge, the ILT field isolate caused infection at less concentrated dilutions and lower EID_{50} titers than the current USDA challenge virus. The ED_{80} estimate corroborates using the less concentrated dilution of the ILT field isolate.

Not all birds that had positive trachea scores exhibited clinical signs or mortality. This is not unexpected due to the ability of ILT virus to replicate in the trachea without exhibiting clinical signs (4). Surprisingly the ILT field isolate produced upper tracheal lesions in 100% of birds at a 1:100 dilution (EID_{50} of $1 \times 10^{0.5}$) and 80% of birds at a 1:250 dilution (EID_{50} of $1 \times 10^{0.1}$). The percent of birds with positive lower trachea scores for the 1:100 and 1:250 dilutions were 80%. At that same 1:250 dilution, only 40% of birds were positive based on clinical signs and mortality, indicating that the ILT field virus infects the trachea without producing clinical signs or mortality.

All of the ILT vaccines currently licensed in the U.S. have label claims for the "prevention of laryngotracheitis", not for "the prevention of infection" (22). The ILT

challenge study, as codified, is designed for a vaccine to receive the "prevention of disease" claim by basing protection on prevention of clinical signs. Several studies indicate that chickens vaccinated with recombinant ILT vaccines may be asymptomatic while challenge or field strain ILT viruses replicate in the trachea (4, 7, 8). Our study supports this, in the fact that we had several birds that were asymptomatic, yet still had tracheal lesions for ILT.

Having a field isolate as vaccine challenge virus would benefit vaccine challenge studies by better replicating what chickens will need vaccine protection from in the field. Also, a new ILT challenge virus that is representative of current field strains is clearly needed in light of the recent ILT outbreaks that have occurred in vaccinated flocks. The ILT field isolate virus used for this study not only produced clinical signs and tracheal lesions similar to those seen with the USDA challenge virus, it could be diluted further then the USDA challenge virus to achieve the same results. Based on tracheal scores, severity of clinical signs and mortality, and the ED_{80}, the optimal dose for the field isolate appears to be a 0.5 ml intratracheal inoculation between dilutions 1:10 and 1:20 (EID_{50} of $1 \times 10^{1.5}$ and $1 \times 10^{1.2}$). All of the information garnered from this work indicates that the field isolate is a viable candidate for use in vaccination challenge studies.

Note - D. Koski's role in journal article and research: First author on journal article. Prepared research proposal and submitted to supervisor for approval. Performed inoculation of chickens. Observed chickens twice daily for ten days. Euthanized chickens and collected all tracheas. Coordinated sample submission to NVSL's pathology laboratory. Worked with CVB Statistics section to analyze data.

Symptom	Score	Definition
Dyspnea	0	Normal Breathing
	1	Mild, open mouth breathing
	2	Gasping with extended neck
Conjunctiva	0	Normal
	1	Swollen and/or partially closed eyes
	2	Eyes completely swollen shut
Depression	0	Normal
	1	Mildly depressed, lethargic, will move when startled but not quickly
	2	Severely depressed, not willing to move, or will only move for a short time when startled
Hemorrhage	0	No presence of blood on beak, head or chest
	2	Presence of blood on beak, head or chest
Mortality (Death)	0	Alive
	2	Dead

Table 1: Scoring of clinical sign severity. Modified from Oldoni et al., 2009.

Score	Histology
0	**Normal**. Epithelium consists of thin pseudostratified columnar epithelium. Mucous glands normal.
1	**Minimal changes**. Normal to slightly thickened epithelium with minimal to mild goblet cell or epithelial cell proliferation; minimal to moderate infiltration of lymphocytes; heterophils rare. No syncytia or cells with intra-nuclear inclusion bodies present.
2	**Mild changes**. Mucosa thickened because of mild to moderate cell infiltration and/or epithelium essentially normal except for foci of syncytia with intranuclear inclusion bodies. Mucosa may contain groups of epithelial cells that have clustered nuclei or are separating from the underlying mucosa. Mucosa may be lined by attenuated epithelium with minimal inflammation and/or decreased goblet cells. Mucosa may be lined by proliferative layers of nonciliated epithelium which contain cyst-like structures. Hyperemia is often present.
3	**Moderate changes.** Mucosa thickened because of moderate to marked cell infiltration. Numerous syncytia with intranuclear inclusion bodies. Patches of affected epithelium often separating from, or, less commonly, sloughed from lamina propria. Mucosal surface well covered by normal or affected epithelium. Mucous glands reduced. Marked hyperemia, cuffs of mono-nuclear cells around vessels outside mucosa.
4	**Severe changes.** Mucosa thickened because of edema, proteinaceous fluid, cellular exudate, or adherent fibrinohemorrhagic to cellular pseudomembrane on the surface. Normal epithelium absent, mucosal surface covered by a thin layer of basal cells. Syncytia with inclusion bodies sometimes present.
5	**Very severe changes.** Same as 4, except mucosa has no residual epithelium and syncytia with inclusion bodies rarely found.

Table 2: Criteria for histopathological scoring of tracheas. Modified from Oldoni et al., 2009.

Treatment	Dilution	Dose (EID_{50})	Classification			% Positive
			Negative	Morbidity	Mortality	
TPB	Negative	NA	10	0	0	0
Challenge Virus	1:4	$1 \times 10^{2.6}$	3	10	7	85
Field Isolate	1:4	$1 \times 10^{1.9}$	2	5	3	80
Field Isolate	1:10	$1 \times 10^{1.5}$	2	7	1	80
Field Isolate	1:20	$1 \times 10^{1.2}$	1	6	3	90
Field Isolate	1:50	$1 \times 10^{0.8}$	2	4	4	80
Field Isolate	1:100	$1 \times 10^{0.5}$	5	3	2	50
Field Isolate	1:250	$1 \times 10^{0.1}$	6	3	1	40

Table 3: Summary of morbidity, mortality and positive percentage of chickens in treatment groups.

Treatment	Dilution	% Severe Lesions Upper Trachea	% Positive Upper Trachea	% Severe Lesions Lower Trachea	% Positive Lower Trachea
TPB	Negative	0	0	0	0
Challenge Virus	1:4	35	95	35	85
Field Isolate	1:4	40	90	30	70
Field Isolate	1:10	20	90	20	80
Field Isolate	1:20	40	100	30	70
Field Isolate	1:50	50	90	50	80
Field Isolate	1:100	20	100	20	80
Field Isolate	1:250	20	80	20	80

Table 4: Summary of upper and lower trachea pathology scores. Severe tracheal lesions are those with scores 4 or 5. Positive tracheas are those with scores of 2 or higher.

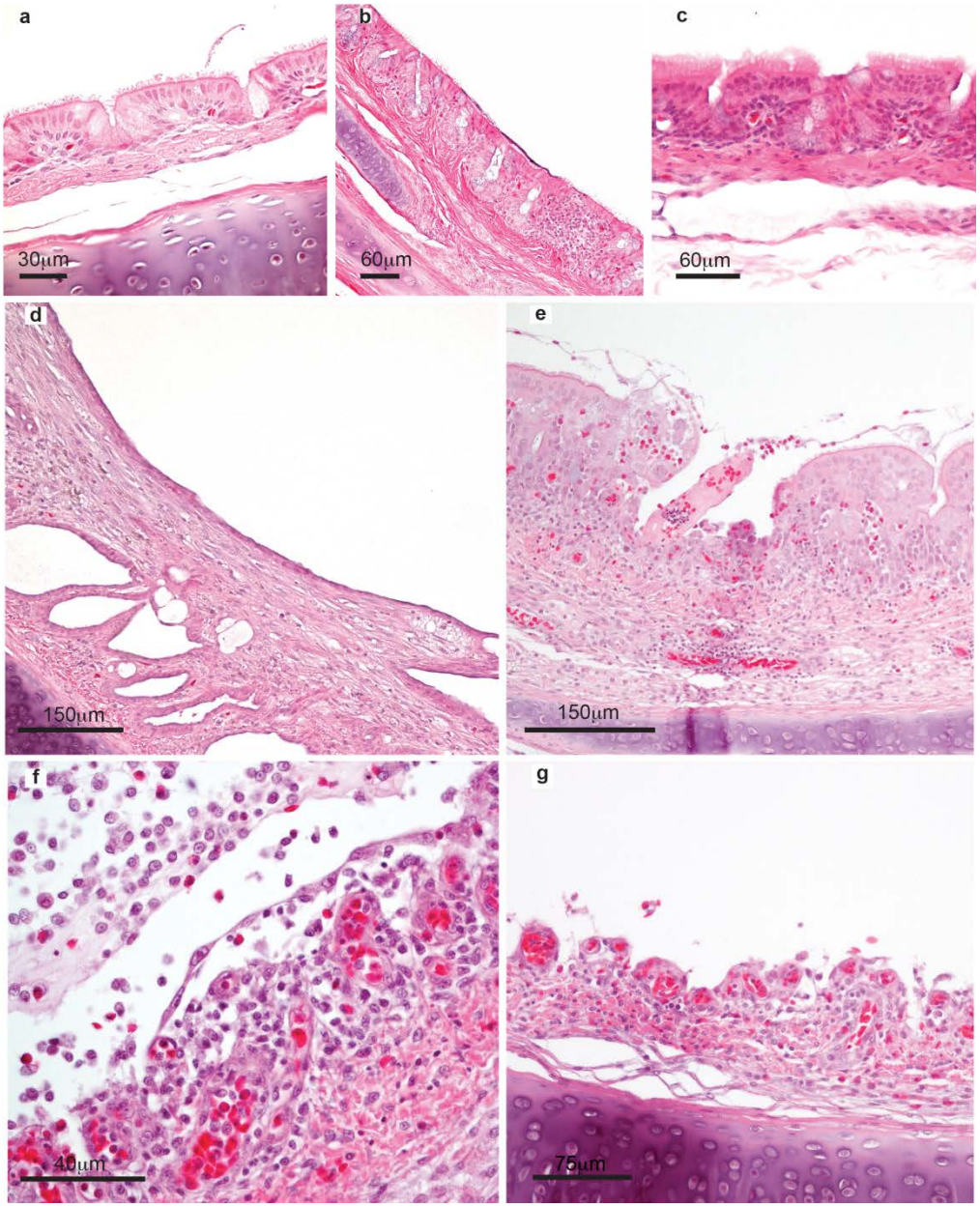

Figure 1. Representative microscopic tracheal lesions and scores in ILTV infected chickens and non-infected controls. 1a: Normal thin pseudostratified columnar epithelium; score 0. 1b: Mild epithelial and goblet cell hyperplasia and hypertrophy; score 1. 1c: Epithelium containing clusters of nuclei separating from the mucosa; score 2. 1d: Proliferative epithelium forming cyst-like structures during regeneration; score 2. 1e: Mucosa thickened because of moderate to marked inflammatory cell infiltration; numerous syncytia with intranuclear inclusion bodies; score 3. 1f: Incomplete thin basal covering; score 4. 1g: Ulcerated mucosa; score 5.

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CHAPTER 3. DISCUSSION: THE COMPLEXITY OF CONTROLLING INFECTIOUS LARYNGOTRACHEITIS DISEASE

General Conclusions:

In 1996, ILT was listed as a reportable disease in at least 16 States (1). In 2010, 1.8 % of breeder chicken farms in the United States reported ILT disease (2). The only respiratory disease reported with more prevalence on those breeder farms in 2010 was *Mycoplasma synoviae* (2). In 2011, in the Delmarva region of Delaware, Virginia and Maryland, 87 farms had outbreaks of ILT (3).

ILT prevention is an important, but complex problem for the poultry industry. Live, attenuated vaccines can revert to virulence (4-6). Through normal farm procedures, chickens shedding live vaccine can infect naïve chickens (7). Recombinant vaccines expressing one or two glycoproteins of ILTV in a herpesvirus of turkeys or fowl pox vector do not revert to virulence, but allow wild type virus to infect, which can lead to outbreaks at a later date (8, 9).

Traditional CEO ILT vaccines are often composed of genetic subpopulations known as quasispecies (10). This is important in relation to the USDA ILT challenge virus which derived from a vaccine strain and has been maintained by passage in eggs. The USDA ILT challenge virus used to test vaccine efficacy was recently found to have become attenuated, requiring increased inoculation concentration to achieve virulence (11). Genetic variation may have allowed attenuation of the challenge virus after serial passage in embryonated eggs.

Environmental pressures can change the population structure of a herpes virus quasispecies. Herpes Simplex Virus type-1 (HSV-1) was found to have genetically

variable populations in intra-ocular fluid of patients with herpes virus infection in the eye (12). The populations were also patient diverse and dependent, meaning each patient had a different quasispecies population. HSV-1 is often treated with the antiviral drug acyclovir (ACV) (12). However, some strains of HSV-1 are resistant to ACV. It is suspected that ACV use shifts the viral population in patients toward ACV resistance (12).

A similar situation has been shown to exist in Marek's Disease Virus (MDV) and the attenuation of virus by cell passage. By full genomic sequencing, it was determined that MDV undergoes genetic changes during passage in cell culture, including gene deletions in virulence factors as passage in cell culture increased (13). Between passes 30 and 40, the virus lost its ability to cause paralysis, and after pass 80, the virus could no longer cause neoplastic lesions in nerves (13). However, there was always genetic variation in the population, and the genetic changes were never in 100% of the population (13). This could mean that upon passage in chickens, genetic variants of an avirulent population may revert to virulence.

Another study using bacterial chromosomes to clone very virulent MDV examined the role of quasispecies in virulence (14). The very virulent field isolate used in the cloning experiments caused cytolytic disease in over 90% of infected birds (14). The clones of the very virulent strain could only cause the same percentage of disease in birds if inoculated together. None of the individual clones could cause the same level of disease, and one clone did not cause any disease symptoms. Virulence appears to be dependent on genetic variation of the quasispecies (14).

The idea of quasispecies in herpes viruses resonates in the context of the challenges of controlling ILT. If the CEO vaccines, as indicated in research, are composed of a mixed population of virus, back passage through chickens could selectively increase the virulent genetic variants. The recombinant vaccines allow ILTV replication *in-vivo*, which in turn may allow the amplification of virulent quasispiecies. Alternatively, replication may increase genetic variation from replication, thus increasing virulence.

Quasispecies may also factor into challenge virus selection. For vaccine manufacturers to produce effective against ILTV, they need a challenge virus representative of field strains that chicken flocks may encounter. A recently isolated, virulent field strain may be an appropriate challenge virus (11). Genetic analysis of the field isolate could determine whether it exists as a quasispecies.

It may be wise to have a quasispecies challenge virus instead of a purified virus strain. Based on MDV research, the genetic variation of quasispecies may be what chickens encounter as virulent field strains. Further research on isolates from ILT outbreaks should determine whether quasispecies play a role in ILTV virulence.

Outside of biosecurity measures, vaccination is the most used control measure for ILT prevention, yet itself is a contributing factor in the epidemiology of the disease. Control of ILT in poultry flocks will require increased understanding of ILTV biology and poultry immunology. A better way to control the complex problem of ILT is possible, but it will involve a multi-faceted approach of incorporating research across the herpesvirus family, educating producers, investigating new technologies, and working with biologics producers and regulators.

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